Vol. 75, No. 8

A Single Amino Acid Substitution in Nonstructural Protein 3A Can Mediate Adaptation of Foot-and-Mouth Disease Virus to the Guinea Pig

JOSE IGNACIO NÚÑEZ,[1,2] ERIC BARANOWSKI,[1] NICOLAS MOLINA,[1]† CARMEN M. RUIZ-JARABO,[1]
CARMEN SÁNCHEZ,[2] ESTEBAN DOMINGO,[1] AND FRANCISCO SOBRINO[1,2]*

*Centro de Biología Molecular Severo Ochoa (CSIC-UAM), Universidad Autónoma de Madrid, 28049 Madrid,[1] and
CISA-INIA, Valdeolmos, 28130 Madrid,[2] Spain*

The genetic changes selected during the adaptation of a clonal population of foot-and-mouth disease virus (FMDV) to the guinea pig have been analyzed. FMDV clone C-S8c1 was adapted to the guinea pig by serial passage in the animals until secondary lesions were observed. Analysis of the virus directly recovered from the lesions developed by the animals revealed the selection of variants with two amino acid substitutions in nonstructural proteins, $I_{248}{\rightarrow}T$ in 2C and $Q_{44}{\rightarrow}R$ in 3A. On further passages, an additional mutation, $L_{147}{\rightarrow}P$, was selected in an important antigenic site located in the G-H loop of capsid protein VP1. The amino acid substitution $Q_{44}{\rightarrow}R$ in 3A, either alone or in combination with the replacement $I_{248}{\rightarrow}T$ in 2C, was sufficient to give FMDV the ability to produce lesions. This was shown by using infectious transcripts which generated chimeric viruses with the relevant amino acid substitutions. Clinical symptoms produced by the artificial chimeras were similar to those produced by the naturally adapted virus. These results obtained with FMDV imply that one or very few replacements in nonstructural viral proteins, which should be within reach of the mutant spectra of replicating viral quasispecies, may result in adaptation of a virus to a new animal host.

The quasispecies structure of RNA viruses endows these pathogens with important biological properties such as adaptability to new environments through rapid selection of mutants from their heterogeneous populations (16, 25). The possibility of acquisition of a new host range and altered tropism and virulence are features of viral quasispecies, highlighted by the emergence of new viral diseases over the last decades, often associated with mutant forms of previously described viruses (16, 31, 37).

Foot-and-mouth disease virus (FMDV) is an aphthovirus that belongs to the *Picornaviridae* family and causes one of the most important animal diseases worldwide (3, 38). The natural FMDV hosts are domestic and wild artiodactyls, mainly cattle, swine, goats, and sheep (3, 38). The open reading frame of the FMDV genome is divided into four separate regions (Fig. 1): L encodes a viral protease, P1 encodes the capsid proteins, and P2 and P3 encode several precursors and a total of nine mature nonstructural proteins. Each of these nonstructural proteins is involved in multiple functions needed for RNA genome replication and particle formation in infected cells (reviewed in references 8 and 39).

Like other RNA viruses, FMDV exhibits a high potential for variation and adaptation, which is reflected in its serological diversity, broad host range, and capacity to produce persistent infections in host animals as well as in cell culture (reviewed in references 18 and 44). There is evidence of multiple virulence

and cell tropism determinants of FMDV in cell culture (4, 5, 9, 27, 28, 32, 40). However, little is known about the virulence and host range determinants of FMDV in vivo. Deletions in nonstructural protein 3A were associated with attenuation for cattle of FMDV serotypes O and C (22). Interestingly, an overlapping deletion in 3A contributes to high virulence for swine of a variant of FMDV serotype O isolated during a recent devastating epizootic in Taiwan (7). There is a need to understand the molecular mechanisms involved in changes of virulence or host range of FMDV, not only because of the great economic impact of FMD but also because the virus is considered a potential zoonotic emergent human disease by some authors (6, 49). Adaptation of FMDV to the guinea pig has been practiced for many decades by vaccine manufacturers in an attempt to derive a manageable virus-host system to study immune responses and vaccine efficacies as an alternative to natural hosts (2, 10, 29). Inoculation of natural FMDV isolates into guinea pigs generally does not produce clinical symptoms. However, the virus can be experimentally adapted to this host by intradermal injection into the footpad and by serial passaging in guinea pigs of homogenates from tissue collected around the inoculation point (2). Following a number of such passages, which varies depending on poorly known influences (site of inoculation, viral strain, individual host, etc.), vesicular lesions appear first at the point of inoculation (primary lesion) and then in the other feet (secondary lesions) (29). This is considered evidence of adaptation to the new host. In spite of the extensive use of guinea pig-adapted viruses in FMDV research, there is no information on the molecular basis of the adaptation to this host.

In this report we identify the genetic changes associated with adaptation of a type C FMDV isolate (clone C-S8c1) to the guinea pig.

* Corresponding author. Mailing address: Centro de Biología Molecular "Severo Ochoa", Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain. Phone: 34-91-3978307. Fax: 34-91-3974799. E-mail: fsobrino@cnb.uam.es.
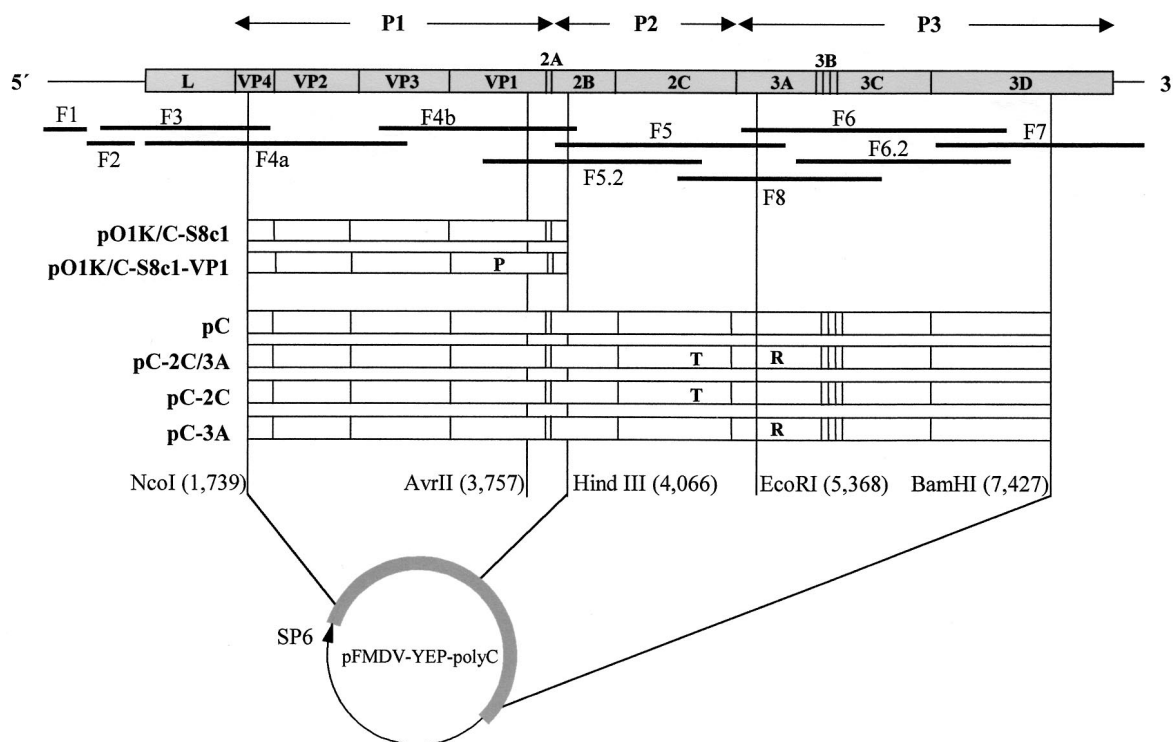† Permanent address: Department of Biology, Universidad del Atlántico, Barranquilla, Colombia.

FIG. 1. Schematic representation of FMDV genome, and strategies for RNA genomic sequencing and for construction of the chimeric plasmids used in this study. Thick lines (F1 to F8) indicate the cDNA fragments used for sequencing of FMDV C-S8c1 genomic RNA, as described in the legend to Fig. 2. Open boxes represent the genomic regions of FMDV C-S8c1 (serotype C) that were replaced in plasmid pFMDV-YEP-polyC, which contains a full-length cDNA of FMDV O1K (serotype O) (shaded boxes) (52). To construct plasmid pC, the C-S8c1 genomic region spanning nucleotides 3573 to 8115 was retrotranscribed and PCR-amplified into four overlapping fragments (fragments F5.2, F6.2, F7, and F8, Table 1). To assemble the four fragments (45), equimolar amounts of each purified fragment were mixed and amplified, using 2.5 U of AmpliTaq Gold (Perkin-Elmer) and 0.12 U of *Pfu* (Promega) DNA polymerases, by 5 cycles of PCR in the absence of primers followed by 30 cycles in the presence of the oligonucleotides hybridizing with the 5′ end of fragment F5.2 and the 3′ end of F7. The resulting 4.5-kb fragment was digested with *Avr*II and *Bam*HI and used to replace the corresponding region (genomic positions 3757 to 7427) in plasmid pO1K/C-S8c1 (5). In the chimeric plasmid pO1K/C-S8c1 (5), the type C FMDV region spans residues 1739 to 4066, which correspond to protein residues S_{33} of VP4 to K_{62} of 2B. In chimeric plasmid pC, constructed in this work, the type C FMDV region spans residues 1739 to 7427, which correspond to protein residues S_{33} of VP4 to W_{283} of 3D. The restriction sites used for these constructions are indicated, and their numbering refers to the C-S8c1 genome (20, 47). The amino acid sequence of VP4 is conserved among FMDVs C-S8c1 and O1K, and the 3D region spanning W_{283} to carboxy-terminal A_{480} differs only at residue 303, which is E in FMDV C-S8c1 and G in O1K. To construct plasmid pC-2C/3A, which encoded replacement $I_{248}{\rightarrow}T$ ($U_{5087}{\rightarrow}C$) in 2C and $Q_{44}{\rightarrow}R$ ($A_{5429}{\rightarrow}G$) in 3A, fragments F5.2, F6.2, and F7 were amplified from C-S8c1 genome and assembled with fragment F8 (Table 1) derived from viral RNA from animal 2.7b. The resulting fragment was cloned into pO1K/C-S8c1, as in the construction of pC. Plasmid pC was used to introduce the single replacement $Q_{44}{\rightarrow}R$ into 3A (plasmid pC-3A). To this end, the *Eco*RI-*Bam*HI fragment (FMDV genomic positions 5368 to 7427) from pC was replaced by the corresponding fragment from pC-2C/3A. Similarly, the *Eco*RI-*Bam*HI fragment from pC-2C/3A was replaced by the corresponding fragment from pC to create plasmid pC-2C, harboring the single replacement $I_{248}{\rightarrow}T$ in 2C. To construct pO1K/C-S8c1-VP1, which encodes mutation $L_{147}{\rightarrow}P$ ($U_{3647}{\rightarrow}C$) in VP1, fragment F4b amplified from viral RNA from animal 2.7b was digested with *Bss*HII and *Avr*II, and the resulting fragment (spanning FMDV genomic positions 3395 to 3757) was used to replace the corresponding fragment in plasmid pO1K/C-S8c1. The introduction of the expected mutations into each of the modified plasmids was confirmed by DNA sequencing, as described in the legend to Fig. 3. The amino acid replacements in plasmid pO1K/C-S8c1 derivatives at positions 147 of VP1, 248 of 2C, and 44 of 3A are indicated.

Adaptation of FMDV to the guinea pig. After four serial passages, only lineage 2 out of four adaptation lineages showed consistent clinical symptoms in inoculated animals (Fig. 2). Primary lesions at the point of inoculation were observed beginning at passage 2, while secondary lesions were noticed in animals 2.4b and 2.6a and in all animals beyond passage 7. In lineages 1 and 4, clinical symptoms were not manifested, while a primary lesion was transiently observed in animal 3.2 (Fig. 2). The virus recovered from animal 2.7b (termed V2.7b) was considered to be adapted to the guinea pig because it consistently led to primary and secondary lesions on further passage in guinea pigs (animals 2.8b to 2.10b in Fig. 2). V2.7b was

chosen for determination of the nucleotide sequence of its genomic RNA.

Characterization of the genomic mutations selected during the adaptation of FMDV to the guinea pig. The nucleotide sequence of the entire genomic RNA directly extracted from a secondary vesicle developed by animal 2.7b was determined and compared with the sequence of the parental FMDV C-S8c1 (Fig. 3). The cDNA fragments used to sequence C-S8c1 genomic RNA are listed in Table 1, and further details are given in the legend to Fig. 1. V2.7b RNA differed from that of C-S8c1 in three nonsynonymous base transitions: $U_{3647}{\rightarrow}C$, $U_{5087}{\rightarrow}C$, and $A_{5429}{\rightarrow}G$. Mutation $U_{3647}{\rightarrow}C$ led to the
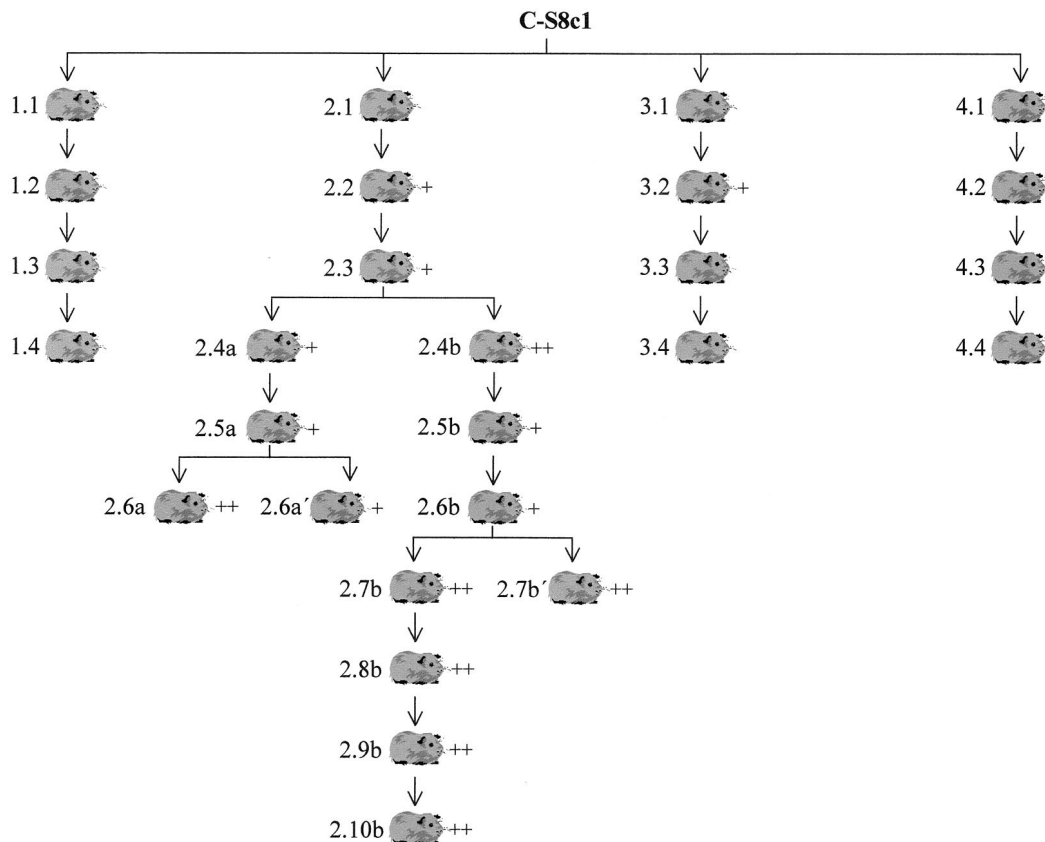
FIG. 2. Scheme of the experiment to adapt FMDV C-S8cl to the guinea pig. Male Dunky Hartley guinea pigs, weighing 250 to 350 g, were inoculated by intradermal injection in the metatarsal pad of the left hind foot with 10^6 PFU of a type C FMDV isolate, C-S8cl, which was isolated from an infected pig in Sta. Pau, Girona, Spain in 1970 (43). After two passages in BHK-21 cells, a plaque-purified, cloned virus was isolated and amplified to about 10^9 PFU to produce the isolate C-S8cl used in this study (43). This virus preparation caused rapid and generalized vesicular disease in swine (12) but not in guinea pigs. Animals were euthanized on day 4 postinfection, the epithelium around the injection point or vesicles (when developed) were excised and homogenized in 0.5 ml of phosphate-buffered saline, and the supernatant was used for successive inoculations (arrows). Animals are designated by lineage number followed by passage number (i.e., animal 2.3 is the animal of lineage 2 at virus passage 3). Sublineages of lineage 2 are indicated by letters (a, a′, b, and b′). Animals which developed visible lesions (vesicles) are indicated as follows: +, development of a primary lesion at the inoculation site; ++, development of a primary lesion at the inoculation site and additional lesion(s) in at least one additional foot (secondary lesion). Note the adaptation of FMDV C-S8cl to guinea pigs in lineage 2 and the transient appearance of a primary lesion in animal 3.2.

$L_{147}\rightarrow P$ replacement, which affected immunodominant B-cell antigenic site A located in the G-H loop of capsid protein VP1 (1, 33); mutation $U_{5087}\rightarrow C$ resulted in replacement $I_{248}\rightarrow T$ at nonstructural protein 2C; mutation $A_{5429}\rightarrow G$ led to replacement $Q_{44}\rightarrow R$ at nonstructural protein 3A. No other synonymous or nonsynonymous replacements distinguished the consensus genomic sequence of the guinea pig-adapted virus from that of C-S8cl.

To study whether the three mutations were fixed simultaneously or sequentially in the course of animal-to-animal passages, RNA was extracted from vesicles produced in 13 animals from lineage 2, the RNA regions where the mutations had been found in V2.7b were amplified by reverse transcription-PCR, and their nucleotide sequences were determined. Mutation $U_{5087}\rightarrow C$ ($I_{248}\rightarrow T$ in 2C) was present in about 10% of the genomes in the first passage (V2.1) and became stably dominant at passage 2 (V2.2 and subsequent passages) (Fig. 3). $A_{5429}\rightarrow G$ ($Q_{44}\rightarrow R$ in 3A) was observed at passage 2 (in about 40% of genomes) and was fully imposed in the next passage

(V2.3 and subsequent passages). $U_{3647}\rightarrow C$ ($L_{147}\rightarrow P$ in VP1) appeared later in the adaptation process and amounted to 40% in V2.4a, 20% in its parallel V2.4b, 50% in V2.5b, and dominant in V2.5a and subsequent passages (compare Fig. 2 and 3). These results indicate a successive fixation of an amino acid replacement in 2C, 3A, and VP1 in the course of adaptation of C-S8cl to the guinea pig. Once imposed, the three mutations were maintained in all viruses recovered from additional passages in guinea pigs (Fig. 3).

The infectivity of viruses V2.3, V2.4b, and V2.10b (Fig. 3) for guinea pigs was confirmed in an independent experiment in which these viruses were used to inoculate two, one, and two new guinea pigs, respectively (data not shown). In all cases, the animals developed lesions as expected while no clinical signs were observed in three animals inoculated in parallel with FMDV C-S8cl. The viral RNA recovered from vesicles showed the mutations present in the parental populations. The presence of substitutions $I_{248}\rightarrow T$ in 2C and $Q_{44}\rightarrow R$ in 3A, but not $L_{147}\rightarrow P$ in VP1, in viruses recovered from the two animals

FMDV	Lesion score	Amino acid		
		VP1 (147)	2C (248)	3A(44)
C-S8 c1		L	I	Q
V 2.1		L	I/T (10%)	Q
V 2.2	+	L	T	Q/R (40%)
V 2.3	+	L	T	R
V 2.4a	+	L/P (40%)	T	R
V 2.4b	++	L/P (20%)	T	R
V 2.5a	+	P	T	R
V 2.5b	+	L/P (50%)	T	R
V 2.6a	++	P	T	R
V 2.6a′	+	P	T	R
V 2.6b	+	P	T	R
V 2.7b	++	P	T	R
V 2.7b′	++	P	T	R
V 2.10b	++	P	T	R

FIG. 3. Amino acid substitutions found in FMDV proteins during adaptation of FMDV C-S8cl to the guinea pig. FMDV isolates are designated by V followed by the animal from which the virus was obtained, using the numbering system described in the legend to Fig. 2. Total RNA from lesions of 13 animals (V2.1 to V2.10b, corresponding to animals 2.1 to 2.10b) were extracted using guanidine thiocyanate (13), and the viral RNA was copied into cDNA and PCR amplified into 11 fragments spanning the whole FMDV genome (Fig. 1), using the oligonucleotide primers shown in Table 1, as previously described (5). The corresponding consensus nucleotide sequences were determined either in an automated sequencer (ABI373) or by using the fmol sequencing kit (Promega). The primers used for nucleotide sequencing have been previously described (5, 47). All regions were sequenced at least twice using primers of opposite polarity (5, 47). Genomic positions are numbered from the 5′-terminal residue, as previously described (20, 47). The nucleotide sequence for the entire genome was determined for V2.7b. For all other viral RNAs, F4b and F5 fragments (Table 1) were copied into cDNA and PCR amplified and the genomic residues spanning, at least, positions 3470 to 3790 (VP1 coding region), 5000 to 5220 (2C coding region), and 5360 to 5630 (3A coding region) were sequenced. The amino acids found at positions 147 of VP1, 248 of 2C, and 44 of 3A are indicated. A percentage in parentheses indicates the proportion of the amino acid that became dominant at later passages, as estimated from the proportion of the intensity of the nucleotide bands corresponding to C_{3647}, C_{5087}, and G_{5429} in the sequencing gels. The lesion score given in the second column is as described in the legend to Fig. 2.

inoculated with V2.3 indicates that these replacements in 2C and 3A in FMDV C-S8c1 were sufficient to produce lesions. However, viruses from the three lesions produced in the animal inoculated with V2.4b included substitution $L_{147} \rightarrow P$ at 60 to 80% in VP1 versus 20% in the virus inoculum (Table 2). This suggests that amino acid replacement $L_{147} \rightarrow P$, although not essential for adaptation of FMDV C-S8c1 to the guinea pig, may confer an additional selective advantage to the virus for replication in the new host.

It must be emphasized that our results do not rule out the possibility that other genetic modifications of FMDV C-S8c1

could also lead to adaptation to the guinea pig. Only one out of four adaptation series led to a C-S8c1 derivative which was virulent for the guinea pig in four adaptation passages, and attempts to isolate virus and to amplify viral RNA sequences from animal 3.2 (which showed transiently a primary lesion [Fig. 2]) failed. Therefore, we are left with a single mutational series of events that resulted in adaptation, and it is likely that C-S8c1, as well as other FMDV clones and isolates, could find alternative pathways for adaptation to the guinea pig, as shown in model studies of fitness gain of FMDV in cell culture (20).

Phenotypic characterization of FMDV C-S8c1 adapted to the guinea pig. Virus V2.3, carrying substitutions $I_{248} \rightarrow T$ in 2C and $Q_{44} \rightarrow R$ in 3A, produced cytopathic effect in BHK-21 and IBRS-2 cells, and the two substitutions were maintained in the viral populations after three passages in BHK-21 cells, suggesting that these changes are not deleterious for replication of the virus in these cells. The viruses recovered were able to produce viral plaques on monolayers of BHK-21 or IBRS-2 with a morphology similar to that produced by the parental FMDV strain C-S8c1 (43). In contrast, inoculation of BHK-21 (four trials) and IBRS-2 (two trials) monolayers with V2.7b or V2.10b, which carried the additional substitution $L_{147} \rightarrow P$ in VP1 (Fig. 3), failed to produce a cythopathic effect, even after serial infections with supernatants of frozen-thawed cell monolayers. Likewise, no cytopathic effect was observed when viruses carrying the VP1 substitution were used to inoculate the following cell lines derived from guinea pigs: colon epithelial cells (ATCC CCL-242), fetal fibroblasts (ATCC CRL-1405), and lung fibroblasts (ATCC CCL-158). Thus, substitution $L_{147} \rightarrow P$ in VP1 abolished productive growth of the virus in different cell lines.

The introduction in an infectious RNA of substitution $Q_{44} \rightarrow R$ in 3A, independently of or in combination with substitution $I_{248} \rightarrow T$ in 2C, renders a virus capable of producing lesions in the guinea pig. An infectious cDNA clone, pC, containing most of the coding region from FMDV C-S8c1, was constructed and used to derive plasmids bearing the mutations found in 2C and 3A in the guinea pig-adapted viruses (details are given in the legend to Fig. 1). The RNA from infectious viruses recovered from BHK-21 cells transfected with transcripts of plasmids pC-2C, pC-3A, pC-2C/3A (harboring substitutions $I_{248} \rightarrow T$ in 2C, $Q_{44} \rightarrow R$ in 3A, or both, respectively), and plasmid pC maintained the expected sequence in 2C and 3A (Table 2). Attempts to recover infective virus, carrying substitution $L_{147} \rightarrow P$ in VP1, in BHK-21 cells transfected with transcripts derived from pO1K/C-S8c1-VP1 that included this replacement (Fig. 1) were unsuccessful, as expected from the lack of infectivity for BHK-21 cells of V2.7b and V2.10b. The only progeny virus obtained carried a unique mutation $C_{3647} \rightarrow U$ in the P1 RNA coding region, which implied a true reversion to restore L_{147} in VP1.

To assess the capacity of pC, pC-2C/3A, pC-2C, and pC-3A to produce lesions in guinea pigs, the viral progenies from transfection of BHK-21 cells were amplified in BHK-21 cells up to a total of 10^8 PFU. Inoculation of guinea pigs with each of the viruses produced primary and secondary lesions in each of the nine animals inoculated with VpC-3A or with VpC-2C/3A but in none of the nine animals inoculated with VpC or VpC-2C (Table 2). In all cases, the mutations present in the parental populations inoculated into the animals were main-

TABLE 1. Synthetic oligonucleotides used for retrotranscription and amplification of FMDV genomes

Fragment[a]	Oligonucleotide sequence (5'→3')	Orientation
F1 (1–367)	TTGAAAGGGGGCGCTAGGGTC	Sense
	GTTAACTGAAAGGCGGGTTTCGGGTG	Antisense
F2 (368–973)	TAAGTTTTACCGTCTGTCCCG	Sense
	AAACCGAGCGCTTTTATAG	Antisense
F3 (569–1643)	CACGATCTAAGCAGGTTTCC	Sense
	CCCTTGAGCTTTCGCT	Antisense
F4a (1002–2835)	CGGAGGTCGGCACCTTTCCTTTAC	Sense
	CTGGGCCAAACCGGCCAAGTAGGT	Antisense
F4b (2744–4189)	CACGAATTCACGGGCAAAGGCTACTGG	Sense
	CAAACGTGCTGTCCAGAATCTC	Antisense
F5 (3988–5699)	TTGGTGTCTGCTTTTGAGGAAC	Sense
	GCCTTCTGACCTGGAAGAGTTC	Antisense
F5.2 (3573–5047)	ACACCGTGTGTTGGCTACGGCG	Sense
	CGCTCACGTCGATGTCAAAGTG	Antisense
F6 (5344–7156)	AAAGGCCAACACGAGGCAGC	Sense
	CGTCGACAATGCGAGTCTTGCCG	Antisense
F6.2 (5678–7156)	GAACTCTTCCAGGTCAGAAGGC	Sense
	CGTCGACAATGCGAGTCTTGCCG	Antisense
F7 (6610–8115)	GGGTTGATCGTTGATACCAGAGA	Sense
	GGATTAAGGAAGCCGGGAAAGCCC	Antisense
F8 (4924–6344)	GGCAAACCCTTCAGCAGTAAG	Sense
	CCAACATCAGCGTTGTTAATC	Antisense

[a] Numbers in parentheses give oligonucleotide positions in the C-S8cl genome, as previously described (20, 47).

tained in the viral RNA recovered from the different lesions analyzed (Table 2). Two out of six animals inoculated with VpC developed a limited lesion at the inoculation point. As expected, the nucleotide sequence obtained from these lesions, corresponding to residues 147 of VP1, 248 of 2C, and 44 of 3A, were identical to those of the parental virus. Thus, the single amino acid replacement Q_{44}→R in nonstructural protein 3A was sufficient to confer on FMDV C-S8c1 the capacity to produce primary and secondary lesions in guinea pigs (Table 2).

Two additional replacements, I_{248}→T in 2C and L_{147}→P in VP1, may also contribute to adaptation to the new host (Fig. 3), but experiments with infectious transcripts suggest that their participation in conferring C-S8c1 virulence for guinea pigs was not essential (Table 2). I_{248} in 2C is only partially conserved among aphthoviruses (data not shown). A positive influence of the L_{147}→P replacement in VP1 on FMDV C-S8c1 replication in guinea-pigs is suggested by the fact that the proportion of virus with this replacement increased from 20% to about 60 to 80% in single animal passages, in the infection with V2.4b. L_{147} is essential for BHK-21 cell recognition by C-S8c1, and replacement L_{147}→P had an adverse effect on such recognition (35), suggesting that P_{147} impairs viral interaction with integrin receptors in BHK-21 cells. Furthermore, L_{147} is conserved among natural FMDV isolates of serotypes O and C (17) and among viruses derived from C-S8c1 in cell culture (11, 14, 42). In addition, previous anal-

yses have shown that replacement L_{147}→P drastically affected the recognition of the antigenic site A by monoclonal and swine polyclonal antibodies (33, 34, 48). This substitution, which became progressively dominant in the course of adaptation of C-S8c1 to guinea pigs (Fig. 3), was lethal for infectivity of C-S8c1 in each of five cell lines tested, including cells derived from guinea pigs, as shown by the inability to produce infectious progeny of the guinea pig-adapted virus and by the recovery of L_{147} revertant mutants from full-length transcripts harboring replacement L_{147}→P.

Little is known about the function of nonstructural protein 3A in the life cycle of FMDV. Information is available for poliovirus, a distantly related picornavirus, in which protein 3A and some of its precursors are involved in virus-specific RNA synthesis in at least two ways. First, 3A includes a single hydrophobic domain (residues 53 to 81) which presumably serves to anchor 3B (VPg) in the membranes, where RNA synthesis takes place (21, 24, 46). Second, 3AB has RNA-binding activity and associates with the 5' cloverleaf of poliovirus RNA and with precursor 3CD to form a ribonucleoprotein complex, which appears to be essential for poliovirus RNA synthesis (24, 50, 51). There is evidence that mutations in 3A may alter the host range and virulence of FMDV and poliovirus in vivo and in cell culture. A 19- and 20-amino-acid deletion in 3A was associated with attenuation for cattle of FMDV O1 Campos and C3 Resende, respectively (22), and an overlapping 10-amino-acid deletion, as well as multiple amino acid replace-

TABLE 2. Lesions developed by guinea pigs inoculated with viruses obtained from infectious FMDV clones

Virus	Animal[a]	Location[b] of lesions on days postinfection:			
		2	3	4	7
VpC	1	—	—	—	(LH)[c,d]
	2	—	—	—	—
	3	—	—	—	—
	4	—	—	—	—
	5	—	—	—	—
	6	—	(RH)[d]	(RH)[c,d]	—
VpC-2C	7	—	—	—	—
	8	—	—	—	—
	9	—	—	—	—
VpC-3A	10	—	LH	LH,[c] RH, LF, RF[c]	
	11	LH	LH, RH	LH,[c] RH,[c] LF,[c] RF[c]	
	12	LH	LH	LH, LF	LH,[c] RH, LF
VpC-2C/3A	13	LH	LH	LH	LH,[c] RH
	14	LH	LH	LH, RH,[c] LF, RF[c]	
	15	LH	LH	LH, RH	LH, RH
	16	LH	LH, RH	LH,[c] RH,[c] LF[c]	
	17	—	LH, RH	LH, RH	LH,[c] RH
	18	—	LH, RH	LH, RH	LH,[c] RH

[a] Guinea pigs were inoculated in the left hind foot with 10^6 PFU (titer determined in BHK-21 cells) of the corresponding viruses obtained from infectious FMDV clones (Fig. 1). RNA transcripts (0.1 to 1 μg), obtained by in vitro translation (5) from the corresponding infectious clone, were introduced into BHK-21 cells using electroporation (27). RNA from chimeric viruses resulting from one round of replication in BHK-21 cells after transfection were retrotranscribed and PCR amplified, and the presence of the expected sequences at the regions spanning the mutated residues (Fig. 1) was confirmed by nucleotide sequencing, as described in the legend to Fig. 3.

[b] Location of the lesions: LH, left hind foot; RH, right hind foot; LF, left fore foot; RF, right fore foot; —, no lesions observed.

[c] The animal showing this lesion was euthanized on the indicated day. Viral RNA was extracted from this lesion and reverse transcription-PCR amplified as described in the text. The nucleotide sequences obtained, corresponding to the regions encoding residues 147 of VP1, 248 of 2C, and 44 of 3A, were identical to those of the parental virus.

[d] Parentheses indicate a small lesion (diameter, ≤3mm).

ments, were associated with the lack of virulence of FMDV O Taiwan 1997 (OTai) for cattle and with impaired plaque formation by this virus in bovine kidney cell monolayers (7). Alignment of 3A sequences shows that these genetic lesions mostly affected positions at the carboxy side of the hydrophobic domain predicted at positions 53 to 81 of 3A (data not shown). Replacement $Q_{44} \to R$ in 3A affects a highly conserved residue among aphthoviruses, located upstream of the hydrophobic domain. Among all sequences available, only that of FMDV C3 Resende and a derivative adapted to chicken embryos, which was attenuated for cattle (22), showed replacement $Q_{44} \to H$ in 3A. In poliovirus, a neighboring mutation $I_{46} \to T$ impaired the cytophatic effect in VERO cells but not in HeLa cells (30). Cell culture-adapted variants of hepatitis A virus also included mutations in 3A (23, 36). Therefore, in a number of picornaviruses, 3A appears to be involved in alterations of host range and virulence, perhaps through modulation of viral RNA synthesis in different cell types.

Comparison of results on the host cell specificity of a number of variants of FMDV, all derived from a single clonal preparation of FMDV C-S8c1, leads to the conclusion that multiple types of genetic modifications result in alterations of cell tropism and virulence. Hypervirulence and cell tropism alterations following cytolytic passages of C-S8c1 have been associated with modifications in the viral capsid (4, 5, 14) whereas hypervirulence for BHK-21 cells of the virus coevolving with cells in persistent infections was associated with a point mutation in the internal ribosome entry site (32). These persistent FMDV strains were attenuated for mice and cattle (15) and did not include replacements in 3A, 3B, 3C, or 3D (47), in contrast to the findings with other attenuated FMDVs (7, 22). Thus, the overall evidence for RNA viruses suggests that there are many host specificity determinants and that variations in nonstructural viral proteins, as well as in RNA regulatory regions and structural proteins, may contribute to the occasional emergence and reemergence of new viral pathogens, in addition to alterations in receptor recognition (41). Such alterations in cell and host specificities may be influenced by the unceasing dynamics of mutant generation and testing in evolving viral quasispecies (16, 19, 26, 31, 37).

REFERENCES

1. Acharya, R., E. Fry, D. Stuart, G. Fox, D. Rowlands, and F. Brown. 1989. The three-dimensional structure of foot-and-mouth disease virus at 2.9 Å resolution. Nature 337:709–716.
2. Aramburu, H. G. 1949. A comparison of different methods of inoculating guinea-pigs with the virus of foot-and-mouth disease. J. Comp. Pathol. 59:42–47.

3. **Bachrach, H. L.** 1977. Foot-and-mouth disease virus, properties, molecular biology and immunogenicity, p. 3–32. *In* J. A. Romberger (ed), Beltsville Symposia in Agricultural Research. I. Virology in agriculture. Allanheld, Osmun, Montclair, N.J.

4. **Baranowski, E., C. M. Ruiz-Jarabo, N. Sevilla, D. Andreu, E. Beck, and E. Domingo.** 2000. Cell recognition by foot-and-mouth disease virus that lacks the RGD integrin-binding motif: flexibility in aphthovirus receptor usage. J. Virol. **74:**1641–1647.

5. **Baranowski, E., N. Sevilla, N. Verdaguer, C. M. Ruiz-Jarabo, E. Beck, E. Domingo.** 1998. Multiple virulence determinants of foot-and-mouth disease virus in cell culture. J. Virol. **72:**6362–6372.

6. **Bauer, K.** 1997. Foot-and-mouth disease as zoonosis. Arch. Virol. **13:**95–97.

7. **Beard, C. W., and P. W. Mason.** 2000. Genetic determinants of altered virulence of Taiwanese foot-and-mouth disease virus. J. Virol. **74:**987–991.

8. **Belsham, G. J.** 1993. Distinctive features of foot-and-mouth disease virus, a member of the picornavirus family: aspects of virus protein synthesis, protein processing and structure. Prog. Biophys. Mol. Biol. **60:**241–260.

9. **Berinstein, A., M. Roivainen, T. Hovi, P. W. Mason, and B. Baxt.** 1995. Antibodies to the vitronectin receptor (integrin alpha V beta 3) inhibit binding and infection of foot-and-mouth disease virus to cultured cells. J. Virol. **69:**2664–2666.

10. **Bittle, J. L., R. A. Houghten, H. Alexander, T. M. Shinnick, J. G. Sutcliffe, R. A. Lerner, D. J. Rowlands, and F. Brown.** 1982. Protection against foot-and-mouth disease by immunization with a chemically synthesized peptide predicted from the viral nucleotide sequence. Nature **298:**30–33.

11. **Borrego, B., I. S. Novella, E. Giralt, D. Andreu, and E. Domingo.** 1993. Distinct repertoire of antigenic variants of foot-and-mouth disease virus in the presence or absence of immune selection. J. Virol. **67:**6071–6079.

12. **Carrillo, C., M. Borca, D. M. Moore, D. O Morgan, and F. Sobrino.** 1998. In vivo analysis of the stability and fitness of variants recovered from foot-and-mouth disease virus quasispecies. J. Gen. Virol. **79:**1699–1706.

13. **Chomzynski, P., and N. Sacchi.** 1987. Single-step methods of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.

14. **Díez, J., M. Dávila, C. Escarmís, M. G. Mateu, J. Domínguez, J. J. Pérez, E. Giralt, J. A. Melero, and E. Domingo.** 1990. Unique amino acid substitutions in the capsid proteins of foot-and-mouth disease virus from a persistent infection in cell culture. J. Virol. **64:**5519–5528.

15. **Díez, J., M. Hofner, E. Domingo, and A. I. Donaldson.** 1990. Foot-and-mouth disease virus strains isolated from persistently infected cell cultures are attenuated for mice and cattle. Virus Res. **18:**3–8.

16. **Domingo, E., C. Biebricher, J. J. Holland, and M. Eigen.** 2001. Quasispecies and RNA virus evolution: principles and consequences. Landes Bioscience, Austin, Tex.

17. **Domingo, E., C. Escarmis, M. A. Martínez-Salas, and M. G. Mateu.** 1992. Foot-and-mouth disease virus populations are quasispecies. Curr. Top. Microbiol. Immunol. **176:**33–47.

18. **Domingo, E., M. G. Mateu, M. A. Martínez, J. Dopazo, A. Moya, and F. Sobrino.** 1990. Genetic variability and antigenic diversity of foot-and-mouth disease virus, p. 233–266. *In* E. Kurstak, R. G. Marusyk, S. A. Murphy, and M. H. V. VanRegenmortel (ed.), Applied virology research, vol. 2. Virus variation and epidemiolgy. Plenum Publishing Corp., New York, N.Y.

19. **Drake, J. W., and J. J. Holland.** 1999. Mutation rates among RNA viruses. Proc. Natl. Acad. Sci. USA **96:**3910–3913.

20. **Escarmis, C., M. Dávila, and E. Domingo.** 1999. Multiple molecular pathways for fitness recovery of an RNA virus debilitated by operation of Muller's ratchet. J. Mol. Biol. **285:**495–505.

21. **Giachetti, C., S. S. Hwang, and B. L. Semler.** 1992. *cis*-acting lesions targeted to the hydrophobic domain of a poliovirus membrane protein involved in RNA replication. J. Virol. **66:**6045–6057.

22. **Giraudo A. T., E. Beck, K. Strebel, P. Augé de Mello, J. L. La Torre, E. D. Schodeller, and I. E. Bergman.** 1990. Identification of a nucleotide deletion in parts of polypeptide 3A in two independent attenuated aphthovirus strains. Virology **177:**780–788.

23. **Graff, J., A. Normann, S. M. Feinstone, and B. Flehmig.** 1994. Nucleotide sequence of wild-type hepatitis A virus GBM in comparison with two cell culture-adapted variants. J. Virol. **68:**548–554.

24. **Gromeier, M., E. Wimmer, and A. E. Gorbalenya.** 1999. Genetics, pathogenesis and evolution of picornaviruses, p. 287–343. *In* E. Domingo, R. G. Webster, and J. J. Holland (ed.), Origin and evolution of viruses. Academic Press, Inc., San Diego, Calif.

25. **Holland, J., K. Spindler, F. Horodyski, E. Grabau, S. Nichol, and S. Vande-Pol.** 1982. Rapid evolution of RNA genomes. Science **215:**1577–1585.

26. **Holland, J. J., J. C. de La Torre, and D. A. Steinhauer.** 1992. RNA virus populations as quasispecies. Curr. Top. Microbiol. Immunol. **176:**1–20.

27. **Jackson, T., F. M. Ellard, R. A. Ghazaleh, S. M. Brookes, W. E. Blakemore, A. H. Corteyn, D. I. Stuart, J. W. Newman, and A. M. Q. King.** 1996. Efficient infection of cells in culture by type O foot-and-mouth disease virus requires

28. **Jackson, T., D. Sheppard, M. Denyer, W. E. Blakemore, and A. M. Q. King.** 2000. The epithelial integrin $\alpha_v\beta_6$ is a receptor for foot-and-mouth disease virus. J. Virol. **74:**4949–4956.

29. **Knudsen, R. C., C. M. Groocock, and A. A. Andersen.** 1979. Immunity to foot-and-mouth disease virus in guinea pigs: clinical and immune responses. Infect. Immun. **24:**787–792.

30. **Lama, J., M. A. Sanz, and L. Carrasco.** 1998. Genetic analysis of poliovirus protein 3A: characterization of a non-cytopathic mutant virus defective in killing Vero cells. J. Gen. Virol. **79:**1911–1921.

31. **Mahy, B. W.** 1997. Human viral infections: an expanding frontier. Antiviral Res. **36:**75–80.

32. **Martínez-Salas, E., J. C. Sáiz, M. Dávila, G. J. Belshan, and E. Domingo.** 1993. A single nucleotide substitution in the internal ribosome entry site of foot-and-mouth disease virus leads to enhanced cap-independent translation in vivo. J. Virol. **67:**3748–3755.

33. **Mateu, M. G.** 1995. Antibody recognition of picornaviruses and escape from neutralization: a structural view. Virus Res. **38:**1–24.

34. **Mateu, M. G., M. A. Martínez, E. Rocha, D. Andreu, J. Parejo, E. Giralt, F. Sorbino, and E. Domingo.** 1989. Implications of a quasispecies genome structure: effect of frequent, naturally occurring amino acid substitutions on the antigenicity of foot-and-mouth disease virus. Proc. Natl. Acad. Sci. USA **86:**5883–5887.

35. **Mateu, M. G., M. L. Valero, D. Andreu, and E. Domingo.** 1996. Systematic replacement of amino acid residues within an Arg-Gly-Asp-containing loop of foot-and-mouth disease virus and effect on cell recognition. J. Biol. Chem. **271:**12814–12819.

36. **Morace, G., G. Pisani, F. Beneduce, M. Divizia, and A. Pana.** 1993. Mutations in the 3A genomic region of two cytopathic strains of hepatitis A virus isolated in Italy. Virus Res. **28:**187–194.

37. **Murphy, F. A.** 1994. New, emerging, and reemerging infectious diseases. Adv. Virus Res. **43:**1–52.

38. **Pereira, H. G.** 1981. Foot-and-mouth disease, p. 333–363. *In* E. P. J. Gibbs (ed.), Virus diseases of food animals. Academic Press Inc., San Diego, Calif.

39. **Porter, A. G.** 1993. Picornavirus nonstructural proteins: emerging roles in virus replication and inhibition of host cell functions. J. Virol. **67:**6917–6921.

40. **Sá-Carvalho D., E. Rieder, B. Baxt, R. Rodarte, A. Tanuri, and P. W. Mason.** 1997. Tissue culture adaptation of foot-and-mouth disease virus selects viruses that bind to heparin and are attenuated in cattle. J. Virol. **71:**5115–5123.

41. **Schneider-Schaulies, J.** 2000. Cellular receptors for viruses: links to tropism and pathogenesis. J. Gen. Virol. **81:**1413–1429.

42. **Sevilla, N., and E. Domingo.** 1996. Evolution of a persistent aphthovirus in cytolytic infections: partial reversion of phenotypic traits accompanied by genetic diversification. J. Virol. **70:**6617–6624.

43. **Sobrino, F., M. Dávila, J. Ortín, and E. Domingo.** 1983. Multiple genetic variants arise in the course of replication of foot-and-mouth disease virus in cell culture. Virology **128:**310–318.

44. **Sobrino, F., M. Sáiz, M. A. Jimenez-Clavero, J. I. Núñez, M. F. Rosas, E. Baranowski, and V. Ley.** 2001. Foot-and-mouth disease virus: a long known virus, but a current threat. Vet. Res. **32:**1–30.

45. **Stemmer, W. P. C.** 1994. DNA shuffling by random fragmentation and reassembly: in vitro recombination for molecular evolution. Porc. Natl. Acad. Sci. USA **91:**10747–10751.

46. **Takeda, N., R. J. Kuhn, C. F. Yang, T. Takegami, and E. Wimmer.** 1986. Initiation of poliovirus plus-strand RNA synthesis in a membrane complex of infected HeLa cells. J. Virol. **60:**43–53.

47. **Toja, M., C. Escarmís, and E. Domingo.** 1999. Genomic nucleotide sequence of a foot-and-mouth disease virus clone and its persistent derivatives. Implications for the evolution of viral quasispecies during a persistent infection. Virus. Res. **64:**161–171.

48. **Verdaguer, N., N. Sevilla, M. L. Valero, D. Stuart, E. Brocchi, D. Andreu, E. Giralt, E. Domingo, M. G. Mateu, and I. Fita.** 1998. A similar pattern of interaction for different antibodies with a major antigenic site of foot-and-mouth disease virus: implications for intratypic antigenic variation. J. Virol. **72:**739–748.

49. **Wisniewski, J., and J. Janjowska.** 1969. Asymptomatic infection with foot-and-mouth disease in man. Przegl. Epidemiol. **23:**263–267.

50. **Xiang, W., A. Cuconati, D. Hope, K. Kirkegaar, and E. Wimmer.** 1998. Complete protein linkage map of poliovirus P3 proteins: interaction of polymerase 3Dpol with VPg and with genetic variants of 3AB. J. Virol. **72:**6732–6741.

51. **Xiang, W., K. S. Harris, L. Alexander, and E. Wimmer.** 1995. Interaction between the 5'-terminal cloverleaf and 3AB/3CDpro of poliovirus is essential for RNA replication. J. Virol. **69:**3658–3667.

52. **Zibert, A., G. Maas, K. Strebel, M. M. Falk, and E. Beck.** 1990. Infectious foot-and-mouth disease virus derived from a cloned full-length cDNA. J. Virol. **64:**2467–2473.